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SLAP deficiency decreases dsDNA autoantibody production

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Abstract

Src-like adaptor protein (SLAP) adapts c-Cbl, an E3 ubiquitin ligase, to activated components of the BCR signaling complex regulating BCR levels and signaling in developing B cells. Based on this function, we asked whether SLAP deficiency could decrease the threshold for tolerance and eliminate development of autoreactive B cells in two models of autoantibody production. First, we sensitized mice with a dsDNA mimetope that causes an anti-dsDNA response. Despite equivalent production of anti-peptide antibodies compared to BALB/c controls, $SLAP^{-/-}$ mice did not produce anti-dsDNA. Second, we used the 56R tolerance model. $SLAP^{-/-}$ 56R mice had decreased levels of dsDNA-reactive antibodies compared to 56R mice due to skewed light chain usage. Thus, SLAP is a critical regulator of B-cell development and function and its deficiency leads to decreased autoreactive B cells that are otherwise maintained by inefficient receptor editing or failed negative selection.

Keywords

B cells; Autoimmunity; Autoantibodies

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1. Introduction

It has been estimated that 75% of newly generated B cells and approximately 30% of mature B cells are autoreactive [1]. Thus, multiple mechanisms exist to render these cells functionally unresponsive or tolerant. In the bone marrow, central tolerance silences newly generated autoreactive B cells primarily by receptor editing and to a lesser extent, through clonal deletion [2,3]. In contrast, peripheral tolerance is established via clonal deletion and anergy [4–7]. Yet, autoreactive B cells make it into the periphery and in some instances lead to autoimmune disease. Removal or inactivation of autoreactive B cells through the mechanisms of tolerance is dependent on strength of signal through the BCR [8]. Src-like adaptor protein (SLAP) is a negative regulator of lymphocyte signaling pathways during lymphocyte development [9–15]. SLAP has been shown to influence BCR levels and signaling in developing B cells by regulating ubiquitin-dependent antigen receptor degradation [14,15]. Thus, we tested the hypothesis that increased BCR signaling as a result of SLAP deficiency will enhance tolerance of autoreactive B cells, including low-affinity self-reactive cells, and prevent the production of autoantibodies and the development of B-cell-mediated autoimmune disease.

The sensitization of BALB/c mice with a peptide mimetope for dsDNA causes the development of an anti-dsDNA antibody response that leads to Ig deposition in the glomeruli of the kidneys [16]. The production of DNA-reactive antibodies in the mimetope model is T-cell dependent [17] and SLAP is also expressed in T cells [9,11–13]. Thus, a second model of autoantibody production was employed in which autoantibodies are the consequence of the forced expression of an anti-DNA-reactive Ig heavy chain in all developing B cells [18].

SLAP-deficient mice were crossed with 3H9H/E56R (56R) mice. 3H9 transgenic mice express a BCR heavy chain within the endogenous BCR locus cloned from a hybridoma derived from an autoimmune MRL/lpr mouse, which reacts with ssDNA when paired with most light chains [19,20]. The reactivity of the 3H9 heavy chain with ssDNA can be reduced or eliminated through pairing with “editor” light chains [20]. 56R is a modification of the 3H9 transgene in which an additional arginine increases the affinity, changes the specificity to dsDNA and renders fewer light chains capable of editing reactivity [18,21,22]. The 56R tg-heavy chain predominantly associates with three Vκ light chains in peripheral B cells: Vκ20, Vκ21D and Vκ38C [21,22]. All three light chains decrease DNA reactivity sufficiently to allow exit from the bone marrow, but the extent to which they eliminate autoreactivity varies. Vκ21 completely eliminates DNA reactivity, whereas Vκ20 and Vκ38C are polyreactive and incompletely edit DNA reactivity [23,24]. Interestingly, further skewing of the light chain repertoire to Vκ21 and Vκ38C has been observed in 56R mice on the BALB/c vs. the B6 background, respectively [21,25]. Biased light chain usage is accompanied by strain-specific differences in autoantibody production. BALB/c.56R mice produce anti-dsDNA of the IgM isotype, but little if any of the IgG isotype, with overall anti-dsDNA levels significantly lower than those of B6.56R mice [22]. Thus, we used the B6.56R model to test if SLAP deficiency could prevent the development of autoreactive B cells.

In this report, we show that deficiency in SLAP, which negatively regulates BCR signaling through ubiquitin-mediated degradation [14], decreases dsDNA-reactive autoantibody production in two complementary models. Our study is an important step in understanding molecular mechanisms of pathogenic autoantibody production. In addition, these findings have implications for targeting B-cell development, and specifically ubiquitination of components of the BCR complex, as a strategy to manipulate B-cell development and function to prevent or treat antibody-mediated systemic autoimmune disease.

2. Materials and methods

2.1. Mice

C57BL/6 and BALB/c were bred in house. The generation of SLAP-deficient ($SLAP^{-/-}$) mice has previously been described [11], and these mice have been backcrossed ten generations onto both the C57BL/6 background and the BALB/c background. 3H9H/E56R (56R) mice were a kind gift from Martin Weigert (University of Chicago). $SLAP^{-/-}$ mice on the C57BL/6 background were crossed with 3H9H/E56R mice on the C57BL/6 background to generate $SLAP^{-/-}$ 56R mice. All mice were maintained in specific pathogen free conditions and the National Jewish Health institutional animal care and use committee approved all animal experiments.

2.2. Antibodies

Monoclonal antibodies against the following antigens were purchased from eBioscience: CD1d (1B1); CD2 (RM2-5); CD4 (GK1.5); CD5 (53–7.3); CD19 (eBio1D3); CD21/35 (eBio4E3); CD23 (B3B4); CD45R/B220 (RA3-B2); Foxp3 (FJK-16 s); IgD (11–26); IgG1 (RMG1-1); IL-10 (JES5-16E3) and T-bet (4B10) conjugated to biotin, FITC, PE, PE-Cy7, PB, APC, APC-Alexa Fluor 750. Streptavidin-APC and monoclonal antibodies against the following antigens were purchased from BD Bioscience: IgM (R6-60.2); IgM^a (DS-1); IgM^b (AF6-78); kappa (187.1); lambda 1, 2 and 3 (R26-46) conjugated to FITC, PE or PerCP-Cy5.5. The monoclonal antibody against CD45R/B220 (RA3-B2) conjugated to PB was purchased from BioLegend. A monoclonal antibody against the lambda light chain conjugated to FITC was purchased from Southern Biotech. A monoclonal antibody against the Vλx light chain conjugated to Alexa Fluor 488 or 647 was a kind gift from Mark Schlomchik (Yale University). Supernatant from the 1.209 hybridoma producing anti-idiotype antibody was a kind gift from David Nemazee (The Scripps Research Institute) and was detected using FITC-conjugated anti-mouse IgG1.

2.3. Intracellular staining

Spleen cells were stained for surface antigens, fixed and permeabilized using eBioscience Foxp3 Staining Buffer Set and stained with anti-T-bet. Data were collected on a CyAn flow cytometer (Dako Cytomation) and analyzed using FlowJo software (Tree Star).

2.4. Hybridomas

B-cell hybridomas were generated from LPS-stimulated splenocytes as previously described [26]. Briefly, pooled splenocytes from 3 representative mice of each strain, producing the average/representative amount of anti-dsDNA in their serum, were stimulated with 50 μg/ml

LPS for 3 days and then fused with SP2/0 (murine IL-6) myeloma cells. Cells were incubated at 37 °C in 5% CO_2 for 48 h before starting selection in 0.5 μg/ml azaserine and 14.1 μg/ml hypoxanthine. Cell culture supernatants were tested for antibody secretion as described below. Hybridomas positive for antibody secretion were further characterized for the isotype of the heavy chain and light chain of the antibody, presence of the transgene and dsDNA reactivity by ELISA as described below. Hybridomas positive by ELISA were expanded, lysed and RNA was extracted using Trizol according to the manufacturer's instructions (Invitrogen). RNA was reverse transcribed using the iScript cDNA Synthesis Kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Rearranged Vκ genes were identified by PCR using Vκ21, Vκ38c and Vκ20 [21], Vκ12/13 [27], VκS [28] and L5 [29] forward primers with the Cκ reverse primers [30]. The PCR conditions for first round synthesis using Vκ21, Vκ38c, Vκ20, Vκ12/13, VκS or L5 forward primers with the Cκ reverse first round synthesis primer were: 92 °C for 7 min, 30 rounds of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 40 s, followed by 72 °C for 4 min. One microliter of PCR products from the first round synthesis was then amplified using the following PCR conditions: 92 °C for 7 min, 35 rounds of 94 °C for 30 s, 50 °C for 20 s and 72 °C for 30 s, followed by 72 °C for 4 min. Hybridomas that only amplified using the L5 or Vκ forward primers were sequenced using the Cκ second strand reverse primer at the Molecular Resource Center at National Jewish Health.

2.5. Induction of peptide-induced anti-dsDNA antibodies

Lupus-like autoimmunity was induced using a peptide surrogate for dsDNA as previously described [16,17]. Briefly, 4- to 8-week-old female BALB/c or $SLAP^{-/-}$ BALB/c mice were immunized intraperitoneally with 100 μg of the DWEYSVWLSN peptide on an eight-branched lysine backbone (MAP™; Applied Biosystems, Foster City, CA; peptide on MAP from Anaspec, Fremont, CA) emulsified 1:1 in complete Freund's adjuvant (CFA) containing incomplete Freunds adjuvant (Difco, Lansing, MI) and 10 mg/ml H37 Ra *Mycobacterium tuberculosis* (Difco). Intraperitoneal booster injections of MAP-peptide in IFA were given on days 7 and 14. Serum was collected at indicated times.

2.6. ELISAs

Serum samples or hybridoma supernatants were analyzed for the presence of dsDNA or MAP-peptide antibodies by ELISA, according to published methods [31,16]. Briefly, dsDNA (calf thymus DNA, Sigma-Aldrich, that had been sonicated and phenol-chloroform extracted) or DWEYSVWLSN MAP-peptide was coated onto Nunc-Immuno MaxiSorp™ 96-well plates (Nalge Nunc International) at a concentration of 10 μg/ml in PBS. After blocking with PBS containing 10% FBS (Hyclone) and 0.2% Tween 20 (Sigma-Aldrich), serial dilutions of serum, supernatant from IgM^a or IgM^b producing hybridomas (kind gift from E. Fournier, National Jewish Health) or mouse IgG, IgM, IgG1, IgG2a, IgG2b or IgG3 standards (Zymed Laboratories) were added to the plates and incubated for 90 min. After washing, peroxidase-conjugated anti-mouse IgG(H and L) (Southern Biotech), IgG1 (Caltag), IgG2a (Caltag), IgG2b (Caltag), IgG3 (Caltag), Igκ (Southern Biotech) or Igλ (Caltag) or biotin-conjugated IgM^a (DS-1; BD Pharmingen) and IgM^b (AF6-78; BD Pharmingen) were added for 90 min. Immunoreactive complexes were detected with 3,3′,

5,5′-Tetramethylbenzidine Liquid Substrate, Slow Kinetic Form (Sigma-Aldrich) and were read at 450 nm in a VERSAmax tunable microplate reader (Molecular Devices).

2.7. Statistical analysis

Unpaired two-tailed Student's *t*-tests, a chi-square test and two-way ANOVAs were performed using Prism 5.0 (GraphPad Software). Differences were considered statistically significant for p values < 0.05.

3. Results

3.1. SLAP-deficient mice injected with a dsDNA mimetope do not produce autoantibodies

To test the effects of increased signaling through the BCR, SLAP-deficient and BALB/c mice were sensitized with a peptide mimetope for dsDNA that causes the development of an anti-dsDNA antibody response and leads to Ig deposition in the glomeruli of the kidneys [16]. Despite the production of an equivalent level of anti-peptide antibodies compared to BALB/c controls, SLAP-deficient mice did not produce anti-DNA antibodies upon sensitization with a peptide mimetope of dsDNA (Fig. 1). A caveat to the use of the mimetope model is that production of DNA-reactive antibodies in the mimetope model is T-cell dependent [17]. SLAP is also expressed in T cells and other hematopoietic lineages including dendritic cells, macrophages and natural killer cells [9,11–13, ImmGen, 32]. Therefore, some of the affects of SLAP deficiency in the mimetope model may be B-cell extrinsic. It has previously been shown that after adoptive transfer of splenocytes into RAG2$^{-/-}$ mice followed by immunization with the dsDNA mimetope, despite the presence of DNA-reactive tetramer + cells, anti-dsDNA antibodies were not detected [33]. Therefore, adoptive transfer of SLAP$^{-/-}$ B cells in combination with WT or SLAP$^{-/-}$ T cells into RAG2$^{-/-}$ mice followed by immunization with the DNA mimetope to examine whether the effects of SLAP deficiency on dsDNA antibodies is B-cell intrinsic was unlikely to work.

Thus, we tested the effect of SLAP deficiency in the 56R model in which autoantibodies are the consequence of the forced expression of an anti-DNA-reactive Ig heavy chain in all developing B cells [18].

3.2. SLAP deficiency leads to decreased production of dsDNA-reactive antibodies in 56R mice

In 56R mice on a B6 background, the expression of the anti-dsDNA-reactive BCR heavy chain results in the maintenance of autoreactive B cells and autoantibody production [22,24]. In addition, 56R B cells have been shown to differentiate, class switch and produce anti-dsDNA of the IgG isotype in the absence of T cells [34]. To test our hypothesis that increasing signaling through the BCR complex through SLAP deficiency would decrease the development of autoreactive B cells and/or the production of DNA-reactive autoantibodies in a B-cell intrinsic model, we crossed 56R mice with SLAP-deficient mice. Blood was collected from SLAP$^{-/-}$ 56R mice and controls, and ELISAs were performed to compare the levels of dsDNA-reactive antibodies in serum. SLAP$^{-/-}$ 56R mice had significantly reduced serum levels of dsDNA-reactive antibodies compared to 56R mice (Fig. 2A). However, it was interesting that although total anti-dsDNA levels were lower in the SLAP$^{-/-}$ 56R group,

a subset of these mice retained the ability to make anti-dsDNA antibodies compared to WT and nontransgenic littermate controls. Thus, we sought to further characterize the antibodies produced in this subset of mice. The isotype of the dsDNA-reactive antibodies in mice with similar amounts of anti-dsDNA reactivity revealed that SLAP deficiency significantly decreased class switching to IgG2a (Fig. 2B), the major anti-dsDNA isotype in 56R mice [34]. This was not due to a generalized effect of SLAP deficiency on class switching since we have previously shown that SLAP-deficient mice have similar levels of serum IgM, IgG, IgG1, IgG2a and IgA compared to WT mice [15]. T-bet has been shown to regulate isotype switching to IgG2a in the 56R model [35,36]. The frequency of SLAP$^{-/-}$ 56R B cells expressing T-bet was significantly decreased compared to that of 56R mice (Fig. 2C). These results support the hypothesis that SLAP deficiency decreases autoantibody production and interestingly, demonstrate a role for SLAP in class switching of autoreactive B cells to IgG2a.

3.3. B-cell subsets and κ vs. λ light chain usage are similar between 56R and SLAP$^{-/-}$ 56R mice

To determine whether decreased levels of serum auto-antibodies in SLAP$^{-/-}$ 56R mice were the result of deletion of autoreactive B cells, we performed subset analysis of developing bone marrow B cells. We confirmed previous reports that 56R B cells undergo negative selection as evidenced by a decrease in absolute numbers of B cells in both bone marrow and spleen [22] (Figs. S1A–C). However, further decreases in these numbers were not seen with SLAP deficiency (Figs. S1A–C). In addition, no significant differences were observed between SLAP$^{-/-}$ 56R and 56R mice in the proportions of bone marrow B cells in fractions E (immature B cells) and F (naïve B cells) (Figs. 3A and B). Moreover, similar frequency of circulating B cells (kappa^{+} pre-B cells) and of kappa and lambda light chain usage in IgM^{+} B cells were observed in SLAP$^{-/-}$ 56R and 56R mice (Figs. 3C and D).

Since SLAP deficiency only had minimal effects on B-cell composition in the bone marrow, we assessed whether it altered composition of peripheral B cells. SLAP$^{-/-}$ 56R mice displayed frequencies and absolute numbers of total splenic B220^{+} B cells (Figs. S1B and C) as well as B220^{+} follicular (FO; CD21^{+}CD23^{+}), marginal zone (MZ; CD21^{+}CD23^{-}) and newly formed/transitional (NF/T1; CD21^{-}CD23^{-}) B cells similar to those in 56R mice, but decreased compared to WT and SLAP$^{-/-}$ controls (Fig. 4A). Similar results were observed for Vκ and Vλ light chain usage in splenic B cells (Fig. 4B). The analysis of light chain usage by splenic B cells revealed a significant fraction of cells in which light chain expression could not be detected using commercially available antibodies against κ or λ (Fig. 4B). This population has previously been reported to express the Vλx light chain, another editor light chain that eliminates binding of 56R antibodies to DNA [37]. The expression of Vλx was assessed by flow cytometry using a monoclonal antibody against Vλx (kind gift from M. Schlomchik). SLAP$^{-/-}$ 56R mice had a similar frequency and absolute number of Vλx expressing B220^{+}IgM^{+} splenic B cells compared to 56R mice (Figs. S2A and B). Collectively, B-cell subsets and Vκ and Vλ light chain usage were similar between 56R and SLAP$^{-/-}$ 56R mice, providing further evidence that receptor editing to λ is unlikely to be the mechanism of tolerance responsible for the decreased production of autoantibodies in SLAP$^{-/-}$ 56R mice.

3.4. Reduction of dsDNA-reactive autoantibody production in SLAP$^{-/-}$ 56R mice is not due to heavy chain editing

SLAP deficiency had no significant effect on frequency or number of B cells in the BM or spleen of 56R mice, indicating that the reduction of a specific B-cell subset was not responsible for the differences in serum autoantibodies. Thus, we examined whether SLAP deficiency decreased anti-dsDNA production by editing the 56R heavy chain. The allotype of the transgenic 56R heavy chain knock-in is IgM^a, whereas that of the endogenous B6 heavy chain is IgM^b [22]. Flow cytometric analysis of splenocytes demonstrated that a higher frequency and absolute number of SLAP$^{-/-}$ 56R B cells expressed IgM^a (88.7%; 9.448×10^6) compared to 56R B cells (80.4%; 6.27×10^6) (Figs. S3A and B). However, not all 56R B cells express the IgM^{a+} heavy chain as a result of class switching. Thus, we confirmed the results using the anti-idiotypic antibody 1.209, which recognizes the 56R heavy chain in combination with most light chains [38]. Similar to the increase in IgM^a expressing B cells in SLAP$^{-/-}$ 56R mice, the frequency of idiotype$^+$ B cells tended to be higher in the spleens of SLAP$^{-/-}$ 56R mice (49.5%) compared to 56R mice (45.9%), but this difference was not statistically significant (Figs. S3C and D). Taken together, these results demonstrate that SLAP$^{-/-}$ 56R mice have a similar if not increased frequency and number of B cells expressing an anti-dsDNA heavy chain, and yet the majority of these cells do not produce dsDNA-reactive antibodies. Thus, heavy chain editing appears not to be responsible for the decreased level of anti-dsDNA in SLAP$^{-/-}$ 56R mice.

3.5. SLAP-deficient 56R mice have skewed κ light chain editor usage

SLAP deficiency had no significant effect on the number of B cells, κ vs. λ light chain usage or heavy chain editing. Thus, we examined whether decreased anti-dsDNA levels were the result of increased receptor editing of the kappa light chain for the expression of κ editor chains or an increase in negative selection of non-editor κ light chains. To examine this possibility, hybridomas were generated from LPS-stimulated splenocytes pooled from 3 representative mice of each strain. The characterization of the allotype and isotype of Ig secreting hybridomas supported the flow cytometric and ELISA data, specifically a higher frequency of IgM^a (Table 1) and a reduction of anti-dsDNA clones in SLAP$^{-/-}$ 56R hybridomas compared to the 56R hybridomas (Figs. 5A and B; Table 1). The hybridoma analysis revealed that the decreased number of SLAP-deficient B-cell clones secreting anti-dsDNA was due to a qualitative change in κ light chain repertoire. The light chain repertoire of SLAP$^{-/-}$ 56R hybridomas was biased toward Vκ21D, an efficient editor of anti-dsDNA reactivity (Fig. 5C). In contrast, incomplete editors of anti-dsDNA reactivity, Vκ38C and Vκ20, predominated in 56R hybridomas. The majority of antibodies produced by hybridomas generated from SLAP$^{-/-}$ 56R mice pair Vκ21D with Jκ2. A bias in the use of this light chain combination has previously been reported for the 56R transgene, but how or why this combination predominates is not known [21,23]. Results from the LPS-stimulated hybridomas reveal that altering the light chain repertoire, either through enhancing negative selection of incomplete editor light chains or through increased receptor editing at the κ locus, is the primary mechanism of tolerance that is responsible for decreased anti-dsDNA production. Taken together, these studies demonstrate that SLAP deficiency prevents the production of DNA-reactive autoantibodies in two complementary mouse models.

4. Discussion

In this study, we show that SLAP deficiency leads to the decreased autoantibody production in two complementary mouse models. SLAP deficiency prevents autoantibody production in a mimetope model of autoantibody production. In addition, SLAP$^{-/-}$ 56R mice produce significantly less DNA-reactive autoantibodies compared to 56R mice. This decreased autoantibody production is primarily due to altered light chain usage, either through enhancing negative selection of incomplete editor light chains or through increasing receptor editing at the κ light chain locus, with skewing of light chain usage toward Vκ21, which eliminates binding to DNA. Thus, SLAP deficiency decreases the threshold for tolerance reducing the production of autoantibodies. These results suggest that the role of SLAP in B-cell tolerance is to allow low affinity self-reactive B cells to develop and persist.

SLAP is a negative regulator of BCR signaling that adapts the E3 ubiquitin ligase c-Cbl to BCR, targeting it for degradation [14]. SLAP is highly expressed in immature B cells [15]. Thus, our initial predictions of how SLAP deficiency would affect development and selection of B cells were based primarily on the bone marrow. The original prediction was that SLAP deficiency would enhance negative selection of autoreactive B cells, thus eliminating autoantibodies in 56R mice. In fact, the data show a significant role for SLAP in altering light chain repertoire. However, whether this is due to a modification in developing B cells or to a change in deletion in the periphery is unclear since SLAP is also expressed in peripheral B cells [15]. SLAP deficiency decreased autoantibody production by skewing light chain usage toward an efficient editor of DNA reactivity, Vκ21D in combination with Jκ2. Use of editor light chains in combination with the 56R heavy chain has been shown to be due to extensive receptor editing [21,39], but this is less clear for the Vκ21D Jκ2 light chain. This Vκ21D Jκ2 bias was previously observed in hybridomas from BALB/c.56R mice [40] but was stated not to be due to receptor editing but instead to effects of selection on the light chain repertoire. Witsch and Bettelheim proposed that 56R Vκ21D Jκ2 B cells are generated at an increased rate because fewer rounds of editing are required to reduce or eliminate dsDNA binding, and increased Jκ2 usage is due to the proximity of the Vκ21 segment to the Jκ cluster. In addition, pairing of Jκ1 or 2 with proximal Vκ segment would leave more substrate for subsequent light chain rearrangements [41]. However, why this preferential pairing would only occur in SLAP$^{-/-}$ 56R or BALB/c.56R mice is unclear. Skewing of the light chain repertoire to Vκ21 and Vκ38C in 56R mice on the BALB/c and B6 backgrounds, respectively, is not found in nontransgenic mice [25,42]. To explain this difference, Witsch and Bettelheim [40] proposed that the threshold for tolerance is genetically defined, with the lupus resistant BALB/c background being more stringent than the B6 background. According to this model, SLAP deficiency, through increasing the avidity of dsDNA-reactive antibodies, shifts the light chain repertoire to the complete editor Vκ21, presumably because it increases the avidity of 56R in combination with the Vκ38C, in essence revoking its editor status and preventing its selection into the repertoire. This supports our hypothesis that SLAP deficiency by increasing strength of signal through the BCR in developing B cells decreases the threshold for tolerance induction leading to decreased autoantibody production possibly preventing the development of autoimmune disease.

SLAP deficiency shifted the light chain repertoire of 56R mice from being dominated by Vκ38 to the complete editor Vκ21. Previously, 56R paired with Vκ20 or Vκ38 has been shown to bind not only to dsDNA, but also to phophotidylserine, a Golgi-associated antigen, myelin basic protein, cytochrome *c*, histone, β-galactosidase and insulin [23,24]. In addition, 56R paired with Vκ20 or Vκ38 has been shown to bind thyroglobulin [23]. Thus, it is possible that SLAP deficiency decreases not only dsDNA-reactive autoantibody production, but also production of autoantibodies to a variety of other antigens. However, further experiments are required to prove this.

SLAP is also expressed in T cells [9,11–13], and since DNA-reactive antibody production in the mimetope model is T-cell dependent [17], the effects of SLAP deficiency in this model could be B-cell extrinsic. Thus, the 56R model was employed. In the 56R model, autoantibodies are the consequence of the forced expression of an anti-DNA-reactive Ig heavy chain in all developing B cells [18] that can differentiate, class switch and produce anti-dsDNA of the IgG isotype in the absence of T cells [27]. Similar to the results in the mimetope model, SLAP deficiency decreased autoantibody production in the 56R model. Thus, T cells are not required for the loss of tolerance, but whether SLAP-deficient T cells can enhance or enforce B-cell tolerance is not known. Further studies are required to dissect these non-mutually exclusive mechanisms.

Previously, we have shown that SLAP deficiency leads to the increased development of Tregs, which have enhanced function and suppress the development of inflammatory arthritis [43]. In addition, we have shown that SLAP deficiency enhances negative selection of $CD8^+$ thymocytes specific for cognate antigen and alters the repertoire of the resulting pool of peripheral $CD8^+$ T cells [44]. These studies in addition to the present study demonstrate in multiple models that SLAP deficiency prevents both T cell-mediated and B cell-mediated autoimmunity, indicating that manipulating ubiquitin-dependent regulation of antigen receptor signaling could prevent autoimmune disease.

In conclusion, we showed that deficiency in SLAP, which negatively regulates BCR signaling through ubiquitin-mediated degradation [14], decreases autoantibody production in two murine models. These findings have implications for targeting B-cell development, and specifically the ubiquitination of components of the BCR complex, as a strategy to manipulate B-cell development and function to decrease or eliminate autoreactive B cells that are either maintained as a result of inefficient receptor editing or failed negative selection upon germinal center formation. A current obstacle to B-cell depletive therapies such as anti-CD20 is that once the B cells return they still have the potential to be autoreactive, produce autoantibodies and cause autoimmune disease. This begs the question as to whether inhibition of ubiquitination of components of the BCR signaling complex could enhance negative selection and/or receptor editing and permanently eliminate pathogenic B cells in patients during their immune reconstitution post anti-CD20. Our studies pave the way to future studies where signaling networks downstream of the BCR signaling complex can be identified and targeted to enhance the negative selection of autoreactive B cells and possibly prevent or treat antibody-mediated systemic autoimmune disease. Increased understanding of the mechanism of the ubiquitin-dependent control of B-

cell development and function could lead to identification of new targets for drug discovery, and translate to new therapies for patients with autoimmune diseases such as lupus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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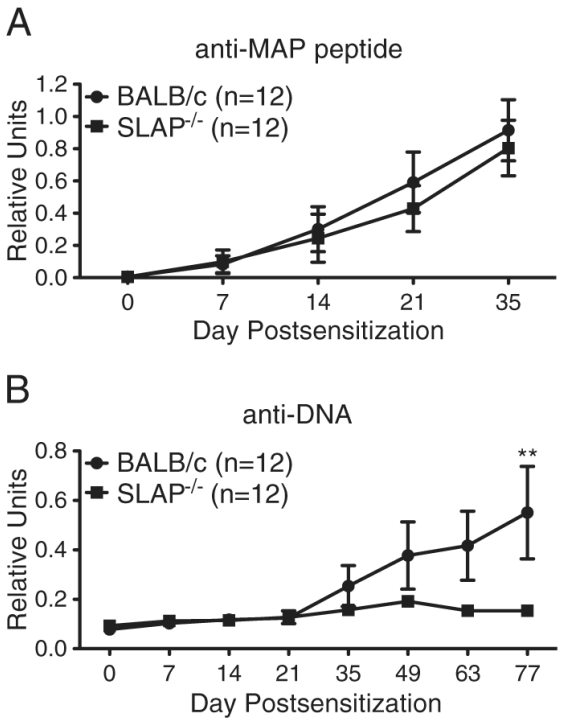

Figure 1.

LAP-deficient mice injected with a dsDNA mimetope do not produce autoreactive antibodies. BALB/c (WT) and SLAP$^{-/-}$ mice were injected with MAP peptide emulsified in CFA and boosted with MAP peptide in IFA on days 7 and 14. Blood was collected at the indicated times and sera were analyzed for antibodies-reactive with dsDNA (A) or MAP peptide (B) by ELISA using an anti-IgG(H + L) secondary antibody to detect all antibodies of the IgG, IgM and IgA isotypes. Data are the average of 12 mice per genotype (±SEM) from three independent experiments. $^{**}p < 0.01$. Statistical analysis was performed using the two-way ANOVA test.

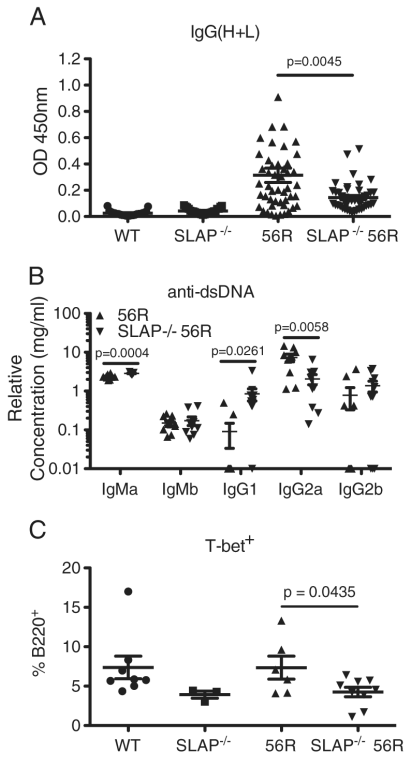

Figure 2.

LAP deficiency decreases anti-dsDNA production in 56R mice. (A) Sera from wild-type (WT; $n = 23$), SLAP$^{-/-}$ ($n = 25$), 56R ($n = 48$) and SLAP$^{-/-}$ 56R ($n = 44$) mice that ranged in age from 1 to 11 months was obtained, diluted 1:500 and analyzed for antibodies-reactive with dsDNA by ELISA. Data are from one experiment. Error bars represent the average (±SEM). (B) Serum from the 10 56R and 10 SLAP$^{-/-}$ 56R mice that produced the greatest amount of dsDNA-reactive antibodies as measured in (A) was further characterized to assess the allotype and isotype of the dsDNA-reactive antibodies. Data are from one experiment. Error bars represent the average (±SEM). (C) Frequency of splenic CD19^{+} B cells that express T-bet. Data represent the average of ten mice per genotype (±SEM) from three independent experiments. Statistical analysis was performed using the two-tailed unpaired *t*-test.

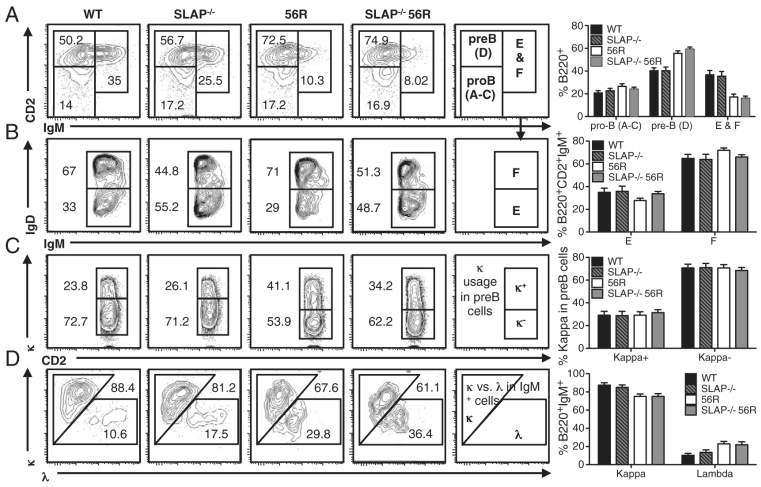

Figure 3.

B-cell subsets in the bone marrow of WT, SLAP$^{-/-}$, 56R and SLAP$^{-/-}$ 56R mice. Bone marrow lymphocytes were gated by forward vs. side scatter and then for B220 expression in WT (n = 14), SLAP$^{-/-}$ (n = 13), 56R (n = 27) and SLAP$^{-/-}$ 56R (n = 37) mice. (A, B) B cells were further subdivided into pro-B, pre-B and fractions E and F based on CD2, IgM and IgD staining. (C) Pre-B cells were analyzed for kappa chain usage by intracellular staining. (D) IgM^{+} cells were analyzed for light chain usage based on kappa vs. lambda staining. Flow plots are representative, whereas graphical data are the average (±SEM) from eight independent experiments.

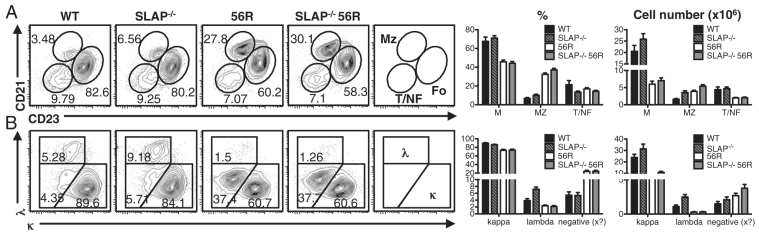

Figure 4.

B-cell subsets in the spleen of WT, SLAP$^{-/-}$, 56R and SLAP$^{-/-}$ 56R mice. Splenic lymphocytes were gated by forward vs. side scatter and then for B220 expression in WT (*n* = 18), SLAP$^{-/-}$ (*n* = 20), 56R (*n* = 32) and SLAP$^{-/-}$ 56R (*n* = 35) mice. (A) B cells were further subdivided into transitional/newly formed (T1/NF), follicular (Fo) and marginal zone (Mz) subsets based on CD21 vs. CD23 staining. (B) IgM^{+} cells were analyzed for light chain usage based on kappa vs. lambda staining. Flow plots are representative, whereas graphical data are the average (±SEM) from twelve independent experiments.

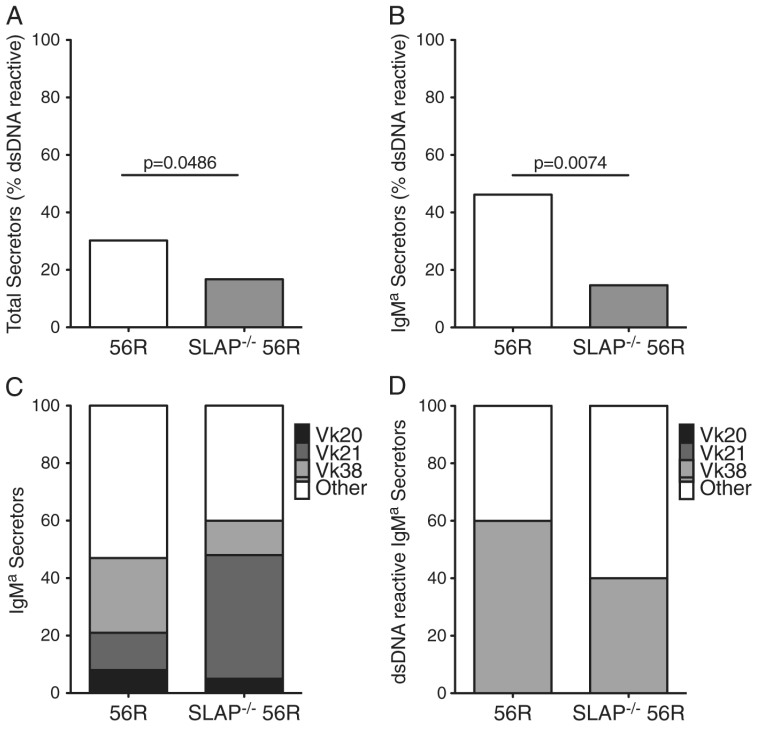

Figure 5.

Biased light chain usage in hybridomas from SLAP$^{-/-}$ 56R mice. Hybridomas were generated from LPS-stimulated splenocytes pooled from three 4-month-old female mice from each strain. Supernatants from 53 56R hybridomas and 50 SLAP$^{-/-}$ 56R hybridomas were screened for dsDNA reactivity (A) and isotype (B) by ELISA. (C, D) RNA isolated from the hybridomas was reverse transcribed and PCR performed on cDNA to determine usage of the three most common light chain editors of anti-dsDNA reactivity. Data are from one experiment.

Table 1

SLAP deficiency enhances receptor editing in 56R mice.

	56R	SLAP$^{-/-}$ 56R
Total secretors	53	50
dsDNA reactive (p = 0.0486)	16 (30.2%)	7 (16.7%)
IgM[a]	26	34
dsDNA reactive (p = 0.0074)	12 (46.2%)	5 (14.7%)
IgM[b]	8	3
dsDNA reactive	0 (0%)	2 (66.6%)
IgG(γ)	10	2
dsDNA reactive	3 (30%)	0 (0%)
Other	9	11
dsDNA reactive	1 (11%)	0 (0%)

Hybridomas were generated from LPS-stimulated splenocytes pooled from three mice of each strain. Supernatants were screened for isotype and dsDNA reactivity by ELISA. Statistical significance was analyzed using a chi-square test.